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Acute exposure to the penconazole-containing fungicide Topas partially augments antioxidant potential in goldfish tissues

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ABSTRACT

Penconazole is a systemic fungicide commonly used in agriculture as the commercial preparation Topas. Although triazole fungicides are widely found in the aquatic environment, little is known about their acute toxicity on fish. In this study we assessed the effects of short-term exposure to Topas on some parameters of homeostasis of reactive oxygen species (ROS), such as the levels of markers of oxidative stress and parameters of the antioxidant defense system of goldfish (*Carassius auratus* L.). Gills appeared to be the main target organ of Topas toxicity, showing the greatest number of parameters affected. Gills of Topas-treated fish showed a higher content of low (L-SH) and high (H-SH) molecular mass thiols and higher activities of superoxide dismutase (SOD), catalase, glutathione reductase (GR), glutathione-*S*-transferase (GST), and glucose-6-phosphate dehydrogenase (G6PDH) as well as reduced carbonyl protein content (CP), as compared with those in the control group. In the liver, goldfish exposure to 15–25 mg L^{-1} Topas resulted in a higher L-SH and H-SH content, but lower CP levels and activity of GST. In kidney, Topas exposure resulted in higher activities of glutathione peroxidase (GPx) and G6PDH, but lower L-SH content and activity of GST. The results of this study indicate that acute goldfish exposure to the triazole fungicide Topas increased efficiency of the antioxidant system in fish gills, liver, and kidney. This could indicate the development of low intensity oxidative stress which up-regulates defense mechanisms responsible for protection of goldfish against deleterious ROS effects.

1. Introduction

Penconazole [1-(2,4-dichloro-β-propylphenethyl)-1H-1,2,4-triazole] belongs to the group of systemic triazole fungicides commonly used in the horticultural, agricultural, and forestry industries for foliar pathogen control (Pose-Juan et al., 2010). It is an active ingredient of many fungicides that are used worldwide such as Topas, Omnex, Oron, Ofir and Dallas. Fungicidal properties are achieved via inhibition of the cytochrome P450-dependent 14α-demethylase activity required in the conversion of lanosterol to ergosterol, an essential component of fungal biological membranes, and disintegration of cellular membranes (Henry and Sisler, 1984; Roberts and Hutson, 1999). At high levels, members of this class of fungicides have a variety of toxicological outcomes in mammals including carcinogenicity, reproductive toxicity, and hepatotoxicity (Zarn et al., 2003; Juberg et al., 2006; Peffer et al., 2007).

These fungicides are normally sprayed directly on plants and are rapidly absorbed and distributed to the interior of the leaves. However, some fraction of the applied pesticides can reach the soil due to drifting during application, rain washing the pesticides off the foliage, and plant material falling onto the soil. Therefore, triazole fungicides constitute a risk to soil ecosystems as well as to ground and surface waters (Kim et al., 2002; Castillo et al., 2006; Kahle et al., 2008; Komárek et al., 2010; Zheng et al., 2016). Penconazole residues have been found in vineyard soils of the Galician province Pontevedra (Northwestern Spain) at a concentration of 50 μg kg^{-1} (Arias et al., 2006). In the Spanish province Ourense, penconazole was also found in soils sampled throughout the year (up to 411 μg kg^{-1}), with the higher concentrations observed in summer and spring during the applications (Bermúdez-Couso et al., 2007). Nevertheless, little is known about penconazole availability in surface water. Indeed, this fungicide was found in surface waters in the Western Cape (South Africa) in concentrations below 2 μg L^{-1} (Dalvie et al., 2003).

In freshwater ecosystems, penconazole can generate adverse effects to aquatic organisms, such as fish. They can accumulate different triazoles rapidly during the 8 days uptake phase, and followed by

Abbreviations: CP, carbonyl protein groups; GPx, glutathione peroxidase; GST, glutathione-*S*-transferase; GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase; H-SH, high molecular mass thiols; LOOH, lipid peroxides; L-SH, low molecular mass thiols; ROS, reactive oxygen species; SOD, superoxide dismutase.

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rapid elimination (Konwick et al., 2006). Penconazole LC_{50}^{96} values for rainbow trout, channel catfish, bluegill sunfish and carp were found to be in the range 1.3–4.6 mg L^{-1} (Surprenant, 1984a, 1984b, 1984c; Rufli, 1984). However, knowledge of the effects of commercial formulations of triazole fungicides on fish is quite limited. Triazoles were found to be potent effectors of cytochrome P_{450} enzymes in rainbow trout (*Oncorhynchus mykiss*) (Levine et al., 1999; Hinfray et al., 2006). Several triazole fungicides depress circulating sex steroid concentrations, thus reducing egg production (Liao et al., 2014; Skolness et al., 2013). Long- and short-term exposure to these fungicides affect both morphological indices and other biochemical parameters in fish, including antioxidant responses, hematological changes, and RNA/DNA ratio (Li et al., 2010a; Li et al., 2013; Zhu et al., 2014).

Therefore, the present study was undertaken to investigate the effects of the industrial penconazole-containing fungicide Topas 100 EC on free radical processes in the gills, liver, and kidney of goldfish (*Carassius auratus* L.).

2. Material and methods

2.1. Reagents

Phenylmethylsulfonyl fluoride (PMSF), 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), oxidized glutathione (GSSG), β-nicotinamide adenine dinucleotide phosphate (NADP), β-nicotinamide adenine dinucleotide reduced (NADH), glucose-6-phosphate (G6P), ethylenediamine-tetraacetic acid (EDTA), xylenol orange, cumene hydroperoxide, ferrous sulphate, 2,4-dinitrophenylhydrazine (DNPH), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), hydrogen peroxide (H_2O_2), NaCl, KH_2PO_4, Tris (hydroxymethylaminomethane), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), pyruvic acid, glutathione reductase from baker's yeast, and β-nicotinamide adenine dinucleotide phosphate reduced (NADPH) were purchased from Sigma–Aldrich Corporation (USA). Topas 100 EC was purchased from Syngenta Crop Protection AG (Switzerland). All other reagents were of analytical grade.

2.2. Animals and experimental conditions

Goldfish (*C. auratus* L.), with body mass of 80–100 g, were obtained from a local fish farm (Halych district, Ivano-Frankivsk region, Ukraine) in October 2014. Fish were acclimated to laboratory conditions for four weeks in a 1000 L tank under natural photoperiod in aerated and dechlorinated tap water. Water parameters were 20 ± 2 °C, pH 7.6–7.8, 7.0–7.6 mg L^{-1} O_2 and hardness (determined as Ca^{2+} concentration) of 39–40 mg L^{-1}. Fish were fed ad libitum with commercial pellets for pond fish "Tetra Pond Sticks" (Tetra, Germany), containing 28% protein, 3.5% fat and 2% cellulose, Zn (48 mg kg^{-1}), Fe (31 mg kg^{-1}), Mn (81 mg kg^{-1}), Co (0.6 mg kg^{-1}), and vitamins A and D_3. Fish were fed during the acclimation period (4 weeks), but were fasted for one day prior and during experimentation.

Experiments were carried out in 120 L glass aquaria (containing 100 L of water), in a static mode, under the same conditions, but with the addition of the commercial fungicide Topas (Syngenta Crop Protection AG, Switzerland) which contains penconazole [1-(2,4-dichloro-β-propylphenethyl)-1H-1,2,4-triazole] at a concentration of 100 g L^{-1}. Groups of five fish were placed in aquaria with different nominal concentrations of Topas fungicide: 1.5, 15 or 25 mg L^{-1}. Topas concentrations used in this work were selected based on an LC_{50}^{96} value (half-lethal concentration after 96 h exposure) for penconazole exposure of carp determined to be 3.8–4.6 mg L^{-1} (Rufli, 1984). Fish were exposed to these conditions for 96 h (no mortality occurred during exposures). Fish in a control group were maintained in the same manner, but without addition of Topas to the water. Aquarium water was not changed over the 96 h course in order to avoid stressing the animals. Levels of dissolved oxygen, temperature and pH were monitored every 24 h. The experiments were carried out in two independent experimental replicates with a total number of at least five biological replicates for every measured parameter.

After exposure, fish were sacrificed by transspinal transsection without anesthesia and tissues (gills, liver, and kidney) were dissected, rinsed in ice-cold 0.9% NaCl, dried by blotting on filter paper, frozen, and stored at −70 °C until use. All experiments were conducted in a strict accordance with the Ethics Committee of Precarpathian National University.

2.3. Determination of oxidative stress indices

Lipid peroxide (LOOH) content was assayed by the FOX (ferrous-xylenol orange) method (Hermes-Lima et al., 1995). Tissue samples were homogenized (1:5, w:v) using a Potter–Elvehjem glass homogenizer in 96% ethanol (4 °C) and centrifuged (5000*g*, 15 min, 4 °C). Aliquots of the supernatants were used for the assay as described previously (Lushchak et al., 2005). The content of LOOH was expressed as nanomoles of cumene hydroperoxide equivalents per gram wet mass of tissue.

Carbonyl groups of proteins (CP) in tissues were determined as described previously (Lushchak et al., 2005). Tissue samples were homogenized (1:10, w:v) in homogenization medium (50 mM potassium phosphate buffer, pH 7.0, 0.5 mM EDTA, 1 mM PMSF) and centrifuged (16,000*g*, 15 min, 4 °C). Supernatants were removed and 0.25 mL aliquots were mixed with 0.25 mL of 40% trichloroacetic acid (TCA) (final TCA concentration 20%) and centrifuged (5000*g*, 5 min, 21 °C). CP levels were measured in the resulting pellets by reaction with 2,4-dinitrophenylhydrazine (DNPH), leading to formation of dinitrophenylhydrazones (Lenz et al., 1989). Values are expressed as nanomoles of carbonyl groups per milligram protein (nmol mg $protein^{-1}$).

Free thiols were measured spectrophotometrically by the Ellman procedure with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 nm (Ellman, 1959). Total thiol concentration (the sum of low and high molecular mass thiols) was measured in supernatants prepared as for the CP assays as described previously (Lushchak and Bagnyukova, 2006). For determination of low-molecular mass thiols (L-SH), aliquots of supernatants were mixed with TCA to reach a final TCA concentration of 10%, centrifuged (16,000*g*, 5 min, 21 °C) to remove pelleted protein and the final supernatants were used for the assay. Thiol concentrations were expressed as micromoles of SH-groups per gram tissue wet mass. The high-molecular mass thiol (H-SH) content was calculated by subtracting the L-SH concentration from total thiol concentration.

2.4. Assay of enzyme activities and protein concentration

Tissue supernatants were prepared as described above for the CP/thiol assays. The activities of antioxidant enzymes including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) as well as the activities of antioxidant-associated enzymes glutathione-*S*-transferase (GST), glutathione reductase (GR), and glucose-6-phosphate dehydrogenase (G6PDH) were measured as described previously (Lushchak et al., 2005).

One unit of SOD activity was defined as the amount of enzyme (per mg protein) that inhibited a quercetin oxidation reaction by 50% of maximal inhibition. Inhibition values for SOD activity were calculated using an enzyme Kinetics computer program (Brooks, 1992). One unit (U) of catalase, GST, GR, GPx, or G6PDH activity is defined as the amount of enzyme that consumed 1 μmol of substrate or generated 1 μmol of product per minute. Activities were expressed as international units (or milliunits) per milligram soluble protein (U mg $protein^{-1}$ or mU mg $protein^{-1}$).

Soluble protein concentrations were measured by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as a standard.